ON THE TRANSGLYCOSIDATION RELATING TO RIBOFLAVIN BY *ESCHERICHIA COLI*

I. FORMATION OF RIBOFLAVINYL GLUCOSIDE

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Recently it has been found (1) in our laboratory that B_2 ¹ and B_2G ¹ play important roles of transglucosidation as revealed by a mutant strain of *Aspergillus oryzae*. Not only B_2G , but also a series of riboflavinyl glycosides, *e.g.*, riboflavinyl-galactoside, -fructoside, -oligosaccharides, and several new flavinyi phosphates, have been isolated in our laboratory from *Asp. oryzae* (2—5) as well as *Escherichia coli*. Thus new transferring reactions of sugar relating to B_2 have been assumed by the authors to be one of the synthetic processes of (1—6) type polysaccharides occurring in bacteria.

In the present paper largely the experiments on B_2G will be described, *i.e.*, the isolation of crystalline B_2G synthesized by dried cells of *E. coli*, the comparison of the crystals of this glucoside with those obtained from rat liver (6) or *Asp. oryzae* (2), and the conditions on the formation of B_2G by the bacterial enzyme.

EXPERIMENTAL

Materials and Methods

Materials — B_2 keto acid, *i.e.*, 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid was synthesized by Surrey's method (7). Lumiflavin was prepared from B_2 by alkaline photolysis, and recrystallized repeatedly from ethanol until B_2 was no more detectable by paper chromatography. Ribityl lumazine, *i.e.*, 8-N-ribityl-6,7-dimethyl-lumazine was kindly supplied from the Research Laboratory of Takeda Co. Ltd. Flavin mononucleotide (FMN) was a commercial product.

Organism — The organism used for this experiment was *Escherichia coli neapolitanus* (B-25). The bacterium was grown in a static culture at 37° in a medium containing 1 per cent peptone, 1 per cent meat extract and 0.5 per cent NaCl. The cells were harvested by Sharples centrifuge, and washed with distilled water. The thin layer of bacterial cells on a Petri dish was dried by an electric fan at room temperature, and kept the dried cells in a dessicator over $CaCl_2$ under reduced pressure. For the experiments for isolating B_2G , the

¹ The following abbreviations are used: riboflavin, B_2 ; riboflavinyl glucoside, B_2G .

dried cells were used.

Preparation of Crude Enzyme—1 g of the dried cells was ground in a porcelain mortar with 5 g of pyrex glass powder and 5 ml of distilled water to a homogeneous paste, and then diluted to 40 ml with water. The suspension was kept for half an hour at 30°, and the supernatant was collected by centrifugation. After being adjusted to pH 7.0, the supernatant was dialysed overnight against 1 l of distilled water. The dialysed solution thus obtained was used for the investigations on the synthesis of B₂G.

Assay method—The total volume of 5 ml of the reaction mixture containing 2 ml of the enzyme, 45 mg of maltose ($2.5 \times 10^{-2}M$), 190 γ of B₂ ($1 \times 10^{-4}M$) and 1 ml of glycine buffer ($1 \times 10^{-1}M$), was adjusted to the requisite pH with 0.2 N HCl or KOH, and was kept in the dark at 30°.

Microdetermination of B₂G was carried out principally by the method of Whitby (8); after the precipitate of enzyme protein was separated by centrifugation, flavin compounds in the supernatant were extracted with 0.25 ml of phenol solution. From the phenol layer, 0.1 ml was applied on the filter paper (Toyo, No. 51) 5 cm in length and developed with an ascending method for about 15 hours with a solvent system of butanol-pyridine-water (6:4:3, by volume). The chromatogram was dried, examined by ultraviolet lamp and the spot corresponding to B₂G (R_F 0.30) was marked.

The spot of B₂G was cut, immersed in 5 ml of distilled water and extracted by heating in a glass-stoppered tube for 15 minutes in the dark at 80°. The solution was cooled and filtered. B₂G in the filtrate was then determined by measuring the optical density at 450 m μ with spectrophotometer. Control experiment using standard B₂ solution showed that the result was satisfactory for quantitative determination.

Results

Isolation of B₂G Synthesized by Dried Cells—100 ml of the incubation mixture containing 2 g of maltose, 40 mg of B₂, 60 mg of flavin mononucleotide (FMN)², 40 ml of glycine buffer (pH 7.0) and 1 g of dried cells suspended in distilled water, was incubated at 30° in the dark for 24 hours, followed by Crammer's procedure (9), and all the flavin compounds were finally extracted with 10 ml of distilled water.

In this extract, 3 or 4 new B₂ compounds migrating to the cathode in paper electrophoresis according to Siliprandi (10), were observed as shown in Fig. 1. The isolation of B₂G was instituted with paper chromatography on a macro-scale, by developing at first with water saturated with isoamyl alcohol and then with butanol-pyridine-water (6:4:3), as described above.

The bands of B₂G on the paper chromatograms were eluted with distilled water by a descending method. The eluate was evaporated to dryness at 45° under reduced pressure, and dissolved in 5 ml of water. The insoluble impurities were removed by centrifugation.

The supernatant was reduced by adding hydrosulfite at 0°, as described by

² FMN was rapidly decomposed into inorganic phosphate and B₂ by the phosphatase of *E. coli*, and the liberated B₂ was then utilized as glycosyl acceptor in the reaction system.

FIG. 1 Two-Dimensional Paper Chromatogram of the Reaction Products from B_2 and Maltose by Dried Cells of *E. coli*.

Reaction mixture containing B_2 , FMN, maltose and dried cells in glycine buffer (pH 7.0) was incubated at 30° for 24 hr. Each spot was detected under ultraviolet lamp. Spot 1 corresponds to B_2G , and spots 2, 3 and 4 to B_2 compounds of oligosaccharide. Chromatogram was developed at first with water saturated with *iso*amyl alcohol, followed by developing with propanol-0.1 *N* NH_4OH (3 : 1).

Masuda (11) for the purification of flavin adenine dinucleotide. The precipitate of reduced B_2G which was collected by centrifugation and washed with a small amount of distilled water, was suspended in 5 ml of distilled water, and then aerated until it was dissolved in the oxidized form. The insoluble residue was removed and the supernatant was concentrated to 2 ml under reduced pressure at 45° .

After several recrystallizations from ethanol at -5° , about 2 mg of orange crystalline powder was obtained. The ratio of optical densities, $375\text{ m}\mu/450\text{ m}\mu$, was found to be 0.86, and the nitrogen content of this crystal corresponded to 93 per cent of the theoretical value.

Moreover, this preparation was found to be identical with the B_2G preparations obtained with rat liver and with *Asp. oryzae* in the characteristics of

TABLE I
Chemical Properties of B_2G

Source of enzyme	<i>E. coli</i> ^a	Rat liver	<i>Asp. oryzae</i>
<i>RF</i> {	B : Ac : W	0.20	0.20
	P : W	0.40	0.40
	W : <i>iso</i> Amyl	0.51	0.50
Ratio of optical densities {	260 $\text{m}\mu/450\text{ m}\mu$	2.24	2.24
	275 $\text{m}\mu/450\text{ m}\mu$	2.03	2.03
	375 $\text{m}\mu/450\text{ m}\mu$	0.86	0.85
mp $^\circ\text{C}$ (with decomposition)	250	247—248	249—250

^a The values obtained in the present experiments

RF values were observed with Toyo filter paper No. 2 using following solvent systems:

B : Ac : W, Butanol-acetic acid-water (4:1:5); P : W, $Na_2HPO_4 \cdot 12\text{ H}_2\text{O}$ -water (5:100); W : *iso* Amyl, water saturated with *iso*amyl alcohol.

ultraviolet absorption spectra and the behaviours of paper chromatography (Table I). It was decomposed at about 250° and turned brown. Glucose was confirmed by paper chromatography of the hydrolysate obtained by heating with 2 N HCl at 100° for 2 hours.

Fundamental Conditions for Synthesis of B_2G —The rate of synthesis of B_2G with the enzyme of *E. coli* was at first linear during 12 hours, and then increased gradually until 20 hours, when the reaction reached to an equilibrium state (Fig. 2). On this state, a secondary reaction in which synthesized B_2G acts as an acceptor of glucosyl, was proceeding. This problem will be discussed in the following paper.

FIG. 2 Formation of B_2G from Maltose and B_2 by the Enzyme of *E. coli*

Reaction mixture (total volume 50 ml) containing $2.5 \times 10^{-2}\text{ M}$ maltose, $1 \times 10^{-4}\text{ M}$ B_2 , 0.1 M glycine buffer (pH 7.0) and 2 ml of enzyme, was incubated at 30° .

FIG. 3 Effect of pH and Temperature on the Rate of B_2G Formation

Reaction mixture (total volume 5 ml) containing $2.5 \times 10^{-2}\text{ M}$ maltose, $1 \times 10^{-4}\text{ M}$ B_2 , 0.1 M glycine buffer and 2 ml of enzyme was incubated for 20 hr.

pH optima were 6.8 to 7.0 (Fig. 3a), and at pH 6.9 (0.1 M glycine buffer), the optimum temperature was about 30° (Fig. 3b). With the glycine buffer higher yield of about 10 per cent than with equimolar citrate buffer was observed, and $2 \times 10^{-2}\text{ M}$ phosphate buffer revealed inhibitory effect on the reaction system. The enzyme was found to be rapidly inactivated on pH below 5.0 at 0° , although it showed considerable stability on an alkaline side.

As for the glucosyl donor, experiments were carried out with various saccharides (Table II). Maltose was found to be the most suitable donor, whereas

TABLE II
Synthesis of B₂G from Various Saccharides

Reaction mixture (total volum 5 ml), containing $5 \times 10^{-2}M$ saccharide, $1 \times 10^{-4}M$ B₂, 0.1M glycine buffer (pH 7.0) and enzyme, was kept at 30° for 20 hr.

Glucosyl-donating substances	B ₂ G synthesized	Ratio of utility ^a
	<i>mM</i>	
Starch	0	0
Dextrin	0.06	22
Glycogen	0.12	43
Maltose	0.28	100
Sucrose	0.08	29
Trehalose	0.13	47
Glucose	0.02	7
Salicin	0.02	7

^a Expressed as percentage of the action with maltose.

glucose was hardly utilized. Therefore, this type of transglucosidation is assumed to belong to the group transfer of D-glucosyl group from maltose to B₂.

Effect of Phosphates and Arsenate — The synthesis of B₂G was inhibited by inorganic phosphate and arsenate. By the addition of phosphate, maltose was partially decomposed phosphorolytically into glucose and glucose 1-phosphate which was confirmed by paper chromatography of two-dimensional method (12). The inhibitory effect by glucose 1-phosphate, as reported by Whitby (8), was again observed in this experiment, and its inhibition was attributable to the competitive effect on glucosyl-donating substances. Glucose 6-phosphate was also inhibitory, but its degree of inhibition was fairly low. It is interesting to note that these inhibiting effects were almost abolished when NaF was added in the concentration of 0.1 M (Table III), as it has been known that NaF inhibits the formation of enzyme-phosphate complex.

TABLE III
Effect of Inorganic Phosphate, Arsenate, Sugar Phosphates and NaF on the Formation of B₂G

Reaction mixture (total vloume 5 ml), containing B₂, maltose and enzyme in glycine buffer as described in Table II, was kept at 30° for 20 hr.

Addition	Concentration	Inhibition
	<i>mM</i>	<i>per cent</i>
Na-phosphate	2	20
Na-phosphate	20	30
Na-arsenate	2	25
Na-arsenate	20	35
Glucose 1-phosphate	25	65
" + NaF	25; 100	20
Glucose 6-phosphate	25	25
" + NaF	25; 100	10

Effect of Uridine Nucleotide — The synthesis of B₂G was not affected by uridine nucleotide, which was prepared from yeast by the method of Caputto

(13), although in the glycosyl-transferring systems of plants and some microorganisms (14, 15), uridine diphosphoglucose was suggested to play a specific role as an intermediate product (Table IV).

TABLE IV
Effect of Uridine Nucleotide on the Formation of B₂G
Experimental conditions were the same as in Table III.

Addition	Concentration	The amount of B ₂ G formed
	<i>mM</i>	<i>mM</i>
Yeast nucleotide	2 (mg)	0.019
" + ATP	2 (mg) ; 0.2	0.019
Uridylic acid	1.0	0.018
" + ATP	1.0 ; 0.2	0.018
None		0.020

Acceptor Specificity—Whitby (8) already given attention to the changes in the degree of glucoside formation by rat liver enzyme according to different isoalloxazine derivatives.

The results of experiments (Table V) using B₂ analogues showed that the transferring reaction was quite specific to the kinds of B₂ analogues as an acceptor, *i.e.*, B₂ keto acid which was prepared from B₂ by removing pyrimidine part of isoalloxazine ring, failed to play any role as an acceptor, since the spots corresponding to the compound of B₂ keto acid and glucose were not detected in paper chromatograms. For ribityl lumazine, which is lacking in the benzene part of isoalloxazine ring, a natural substance produced by mould was used. Any glucosyl compounds of ribityl lumazine were never detected, but ribityl lumazine was found to reveal an inhibitory effect on the synthesis of B₂G. Remarkable inhibition on B₂G formation was observed with lumiflavin, as given in a Lineweaver-Burk plot (16) in Fig. 4, which shows competitive inhibition between B₂ and lumiflavin.

It is suggested that B₂ as a

FIG. 4 *Competitive Inhibition on the Synthesis of B₂G by Lumiflavin*

Curve 1. without lumiflavin. Curve 2. with 1×10^{-4} M lumiflavin. Ordinate ($1/V$): reciprocal value of the amount of formation of B₂G ($1/M/2$ ml enz./20 hr). Abscissa ($1/S$): reciprocal value of final concentration (M) of B₂.

Reaction mixture (total volume 5 ml) was incubated at 30° for 20 hr as was shown in Table III.

TABLE V
Inhibitory Effect of B₂ Analogues on B₂G Formation
 Experimental conditions were the same as in Table III.

Addition	Concentration	Inhibition
	<i>mM</i>	<i>per cent</i>
B ₂ keto acid	0.2	13
Ribityl lumazine	0.2	36
Xanthine	0.4	36
Uracil	0.4	13
Lumiflavin	0.2	82

glucosyl acceptor is activated by the enzyme, producing the B₂-enzyme intermediate in the first step of this transglucosidation. Therefore phenol derivatives such as salicylic and *p*-aminosalicylic acids, which were assumed to inhibit the formation of this intermediate product (17), also revealed strong inhibitory effects on B₂G formation (Table VI).

TABLE VI
Inhibitory Effect of Phenol Derivatives
 Experimental conditions were the same as in Table III.

Addition	Concentration	Inhibition
	<i>mM</i>	<i>per cent</i>
<i>p</i> -Nitrophenol	50	50
Salicylic acid	100	85
<i>p</i> -Aminosalicylic acid	100	55

TABLE VII
Effect of Functional Group Reagents on the Activity of Enzyme
 Experimental conditions were the same as in Table III. The reagents were added to the buffered enzyme solutions and kept for 15 minutes before the addition of substrates.

Reagent	Concentration	Inhibition
	<i>mM</i>	<i>per cent</i>
KCN	5	-9
α α' -Dipyridyl	1	-5
EDTA	1	9
PCMB	0.1	77
"	1	82
PCMB + Cysteine	1;10	18
PCMB + GSH	1;10	14
AgNO ₃	1	100
CuSO ₄	1	73
I ₂	1	100
Cystine	10	50
K ₃ Fe(CN) ₆	1	50
HgI	1	91

Methanol, nitrophenol and sugar alcohol failed to act as direct acceptors of glucosyl to form methyl glucoside, *etc.* Moreover, no noticeable effect of these alcohols was observed on the formation of B₂G.

Effect of Functional Group Reagents—The enzyme of *E. coli* lost its activity easily by oxidation. The effects of mild oxidizing reagents and SH reagents were examined, with the results shown in Table VII.

The enzyme activity was slightly increased by the addition of KCN or α, α' -dipyridyl, and inhibited strongly by heavy metal ions even at low concentrations. The inhibition by *p*-chloro-mercuribenzoate (PCMB) was almost complete at the concentration of $1 \times 10^{-4} M$, but it was reversed satisfactorily by the addition of cysteine or glutathione (GSH).

Effect of Redox Dyes—The relationship between the activity of the enzyme and the redox potential of the reaction mixture was shown in Fig. 5. It will be seen that the maximum activity was observed at the potential -0.19 volt, which is identical to the normal potential of B₂ itself. From this result it is assumed that B₂ may play another role in keeping optimum redox potential for B₂G formation.

FIG. 5 Effect of Redox Dyes on the Formation of B₂G

Reaction mixture (total volume 5 ml) containing maltose, B₂ and enzyme in glycine buffer (pH 7.0) and 0.1 per cent of redox dyes represented below, was kept at 30° for 20 hr.

No. 1, 2,6-dichlorophenol indophenol ($E_0 = 0.217$); No. 2, toluylene blue (0.115); No. 3, methylene blue (0.001); No. 4, indigo disulfonate (-0.125); No. 5, cresyl violet (-0.163); No. 6, phenosafranine (-0.252); No. 7, neutral red (-0.340).

DISCUSSION

The results of the above experiments on the suitable conditions of B₂G formation, have led to the conclusion that the cause of the transferring action is attributable to the combination of isoalloxazine with an active site of an enzyme molecule, especially the combination of the pyrimidine part of isoalloxazine ring with the functional group of the enzyme protein, possibly through a hydrogen bond. It is also assumed that the B₂-enzyme intermediate product thus formed may give rise to the transformation of glucosyl group under a suitable redox potential, whereas inorganic phosphate inhibits the formation of the intermediate product and phosphorolytical decomposition of sugar takes place. Namely, the mechanism of transglucosidation is not a direct combination of monosaccharide to B₂, but a transfer of glucosyl group from maltose to B₂ *via* an enzyme as postulated as follows.

Where E represents the transferring enzyme, B_2E the intermediate product, and (B_2E) glucose the glucosidated intermediate.

This reaction, together with the finding which will be reported in the following papers, explains the continuous transglucosidation to increase the number of glucosyl groups in order to form B_2 compounds of oligosaccharide. Thus this reaction gives a quite new interpretation of the action of B_2 under a suitable redox potential.

There have been many elaborate discussions about group-transferring actions. Recently it has been recognized that a large number of hydrazes catalyzes group transfer among certain donors and acceptors other than water. However, there is no definite conclusion about the relationship between an acceptor and the corresponding enzyme.

The authors have pointed out already the transglucosidation by *Asp. oryzae* relating to B_2 in the previous report (1). These results also confirm the possible existence of B_2 -enzyme intermediate product.

SUMMARY

1. B_2G synthesized from maltose and B_2 by *E. coli*, compared with those obtained by the enzyme of rat liver and of *Asp. oryzae*. The chemical properties of these B_2G are found to be quite identical.

2. For the formation of B_2G , the optimum temperature and pH were found to be 30° and 6.9, respectively, and maltose was chosen as most suitable glucosyl donor among various saccharides.

3. The formation of B_2G was inhibited by both inorganic and organic phosphates, and the latter is attributed to the competitive effect on glucosyl-donating substances. Powerful inhibition revealed by the mild oxidizing reagents and SH reagents is due to the direct action on the enzyme itself.

4. The isoalloxazine ring of B_2 was found to play an important role in this kind of transglucosidation, and the glucosidation was inhibited profoundly by lumiflavin. The redox potential of B_2 is suitable for the glucosidation. The authors have postulated the mechanism of this transglucosidation and discussed on its significant role.

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